Bosentan regulates the expression of adhesion molecules on circulating T cells and serum soluble adhesion molecules in systemic sclerosis-associated pulmonary arterial hypertension.

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Short running title: Adhesion molecules in SSc-PAH.

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ABSTRACT

Objectives To study the expression of adhesion molecules in Systemic Sclerosis (SSc) patients with and without pulmonary arterial hypertension (PAH) and the effects of therapy with the endothelin-1 (ET-1) receptor antagonist, bosentan.

Methods Thirty-five SSc patients and 25 healthy donors (HD) were selected for this study. Ten of 35 patients had isolated PAH assessed by Doppler echocardiography and treated with bosentan. PB lymphocytes were isolated by density gradient centrifugation, and the expression of LFA-1, VLA-4, L-selectin on CD3 T cells was assessed by double immunofluorescence and flow-cytometry. As endothelial activation markers, serum soluble P-selectin, PECAM, VCAM-1, ICAM-1, and vWF antigen were assessed by ELISA. In SSc-PAH patients, T cell subsets and soluble endothelial markers were assessed at baseline and after 6 and 12 months of bosentan therapy.

Results In SSc-PAH patients, serum soluble ICAM-1, VCAM-1, P-selectin, and PECAM levels were higher than in HD at baseline and fell to normal values after 12 months of bosentan therapy. CD3-LFA1 T cells were significantly higher in PAH-SSc at baseline than in HD or SSc and significantly decreased after therapy. CD3-Lselectin T cells were significantly lower in SSc-PAH at baseline than in HD or SSc and rose to normal levels after bosentan therapy.

Conclusions This study confirms that endothelial activation occurs in SSc, and suggests that changes in the T-cell/endothelium interplay take place in SSc-associated PAH. Bosentan seems to be able to hamper these changes and restore T cell functions in these patients.

Key words: ICAM-1, VCAM-1, LFA-1, VLA-4, L-selectin
Short running title: Adhesion molecules in SSc-PAH.
INTRODUCTION

A crucial role in the pathogenesis of systemic sclerosis (SSc) seems to be played by the interactions occurring between endothelial cells and T cells. Endothelial cells, after metabolic activation, produce an array of cytokines and growth factors, and promote immune cells to adhere and migrate through the vessel wall into the extra-vascular space. This leads to fibroblasts and myofibroblasts activation and changes in extra-cellular matrix (ECM) remodelling, resulting in an excessive accumulation of ECM components that causes fibrosis of the skin and internal organs and hypertrophy/hyperplasia of the arterial wall of pulmonary and renal vessels. Circulating peripheral blood T cells adhere to endothelial cells by means of an intricate system of adhesion molecules whose expression increases on the cell surface following activation. LFA-1 (lymphocyte function antigen-1), VLA-4 (very late antigen-4), and L-selectin are expressed on lymphocytes, while their counter-receptors ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1), and CD34/endoglycan are expressed on endothelial cells. When over-expressed on the activated endothelial layer, VCAM-1, ICAM-1 as well as P-selectin and PECAM-1 (platelet endothelial cell adhesion molecule-1) undergo shedding and their soluble forms, sVCAM-1, sICAM-1, sP-selectin and sPECAM-1, respectively, are detectable in serum and considered to be markers of endothelial cell activity or injury. There is a growing body of evidence that these endothelial activation markers are raised in patients with SSc and that their levels correlate with disease activity and can be regulated by therapy.

Endothelin-1 (ET-1) is a potent mitogenic factor mainly produced by endothelial cells, and exerts its biological activity by interacting with two cell membrane-bound receptors named ET\(_{\text{A}}\) and ET\(_{\text{B}}\), expressed on different cells, such as endothelial cells, smooth muscle cells, fibroblasts. ET-1 has pleiotropic functions, being a potent vasoconstricor, and stimulates synthesis and accumulation of ECM proteins by fibroblasts and smooth muscle cells. Since its levels have been found increased in SSc it is believed to have a key role in the pathogenesis of this disease. In particular, ET-1 levels have been detected in lung plexiform lesions in patients with pulmonary arterial hypertension (PAH) and there is evidence, both from experimental models and clinical studies, that ET-1 is directly involved in promoting the remodelling of vessel walls leading to an increase of peripheral vascular resistance and hence a rise in pulmonary arterial pressure. Recently, a dual endothelin receptor antagonist named bosentan has been proven to be effective and safe for the treatment of SSc patients with PAH. In this study, the biological outcome of bosentan on endothelial activity and T cell activation has been evaluated in patients with SSc-associated PAH. Expression of LFA-1, VLA-4, and L-selectin has been investigated on peripheral blood T cells in SSc-PAH patients at baseline and after bosentan treatment. Serum levels of soluble VCAM-1, ICAM-1, P-selectin, PECAM-1, and vWF (von Willebrand Factor) were also assessed.
METHODS

Patients. Thirty-five SSc patients were consecutively recruited at the Rheumatology Unit of Bari. Mean age was 51.4 years (range 27-75) and mean disease duration was 10.4 years (range 1-28). All the patients were assigned to the limited cutaneous subset, according to the Leroy subset classification. Twenty-five healthy donors (HD), coming from the same geographic area and attending the Rheumatology Unit as students, teachers and employers, were enrolled on the basis of their age (mean age 46.7 years, range 25-69). In the SSc patients, high resolution chest tomography was carried out to assess interstitial lung fibrosis, as well as respiratory function tests to evaluate pulmonary forced vital capacity (FVC) and alveolar CO diffusion (DLCO), along with full blood count, renal function tests, serum complement levels, liver enzymes, erythrocyte sedimentation rate (ESR). Ten of 35 patients had isolated PAH assessed by Doppler echocardiography (tricuspid gradient at rest >35 mmHg), and subsequently confirmed by right heart catheterization; mPAP was 38.6 ±9 mmHg, pulmonary vascular resistance was 758 ±254 dyne.sec/cm, pulmonary capillary wedge pressure was 13 ±1.7 mmHg. SSc-PAH patients were in NYHA functional classes II-III according to the WHO classification, with 6-minute walking distance (6-MWD) ranging between 60 and 450 m. No patient had left ventricular disease at Doppler echocardiography. Further characteristics of the SSc patients with PHA are shown in detail in Table 1. SSc-PAH patients were treated with bosentan at the standard dosage of 62.5 mg twice daily for 4 weeks, followed by 125 mg twice daily for 50 weeks. Additional permitted drugs were intestinal prokinetics and H-2 blockers. No patients were on steroids and/or immunosuppressive agents. Clinical assessment, including 6-MWD and Doppler echocardiography, and collection of serum and heparin blood samples were done at baseline, and after 6 and 12 months of bosentan therapy. Written informed consent was obtained from all patients and healthy donors according to the declaration of Helsinki and the study has been approved by the Ethics Committee of the Policlinico di Bari.

T lymphocyte phenotype. Peripheral blood (PB) lymphocytes were isolated by gradient density separation on lymphoprep and cell surface antigen expression on T cells was assessed by double immunoflorescence. Briefly, 5 x 10⁵ cells were incubated with 5 µl of mAb (CD3-PE, CD11a/LFA1-FITC, CD49d/VLA4-FITC, CD62L/Lselectin-FITC, Immunotech, Marseille, France) for 20’ on ice, washed twice and T cell subsets were evaluated by flowcytometry (FACScan, Becton Dickinson). T cell phenotype was evaluated at each time point on freshly isolated lymphocytes against a control sample, incubated with rat IgG1-FITC/IgG2-PE (DAKO, Denmark).

Soluble endothelial activation markers. Serum levels of endothelial markers were assessed by the commercial ELISA, sICAM-1 ELISA kit Cell Com (Cellular Communication Investigation, Immunotech, Marseille, France), sVCAM-1 ELISA kit IBL (Immuno Biological Laboratories, Hamburg, Germany), vWF ELISA kit Gentaur (Brussels, Belgium), sPECAM-1 ELISA kit Bender MedSystems (Wien, Austria), sP-Selectin ELISA kit Bender MedSystems, (Wien, Austria), following the manufacturers’ instructions. ELISA experiments were ran simultaneously on all samples previously frozen at -30°C.

Statistical analysis. Values are expressed as mean ± 1 SD, unless otherwise indicated. Analysis of variance (ANOVA) with the post hoc LSD (least square differences) range test was used to compare the different groups at baseline, whereas to compare SSc-PAH patients at the different time points paired t-test was applied. U-Mann Whitney test was used to compare NYHA functional classes over time. Correlations of clinical data with endothelial activation markers and T cell phenotype were carried out using Pearson’s or Spearman analysis. The significance level was set at p <0.05.
RESULTS

Clinical outcomes. Bosentan treatment significantly improved exercise ability in SSc-PAH patients with 6-MWD, which rose from 267 ± 33 m at baseline to 359 ± 27 m at 6 months and to 376 ± 40 m at 12 months (p<0.05 vs baseline). Bosentan also induced an improvement of dyspnea, measured by NYHA, with a significant reduction of the NYHA functional class, observed after 12 months of therapy (median baseline 2.0 vs median 12 months 1.5, p<0.05). PAP values reduced during bosentan treatment, without reaching statistical significance (data not shown). Digital ulcers were present in 8 out of 10 patients and 6 patients did not experience new digital ulcers during bosentan therapy.

Adhesion molecule expression on T cells. Absolute number of lymphocytes did not change upon bosentan therapy. As shown on Figure 1a, PB T cells expressing LFA-1 were significantly higher in SSc-PAH patients at baseline (46.3% ±6) than in HD (32.6% ±3, p<0.05) or in SSc patients without PAH (35.8% ±5, p<0.05). They decreased after 6 months of bosentan therapy (35.5% ±5, p<0.05 vs baseline) and fell to normal values after 12 months (32.7% ±4, p<0.05 vs baseline). The proportion of T cells bearing VLA-4 antigen (Figure 1b) was significantly reduced in the SSc-PAH group (73.4% ±4) in comparison with HD (83.3% ±6, p<0.05) and SSc patients without PAH (84.8% ±5, p<0.05), but was not regulated by bosentan treatment after 6 months (70.5% ±6, not significant vs baseline) nor after 12 months (75.1% ±2, not significant vs baseline). Expression of L-selectin on T cells (Figure 1c) was significantly lower in SSc-PAH patients (26.4% ±8) than in the HD group (75.5% ±8, p<0.01) or in SSc patients without PAH (75.5% ±2, p<0.05), and gradually rose following bosentan treatment at the 6 months (43.1% ±10, p<0.05 vs baseline) and 12 months controls (63.2% ±6, p<0.01 vs baseline). In Figure 2, the representative histograms of the fluorescence intensity for CD3-Lselectin cell subset from a SSc-PAH patient before and after bosentan therapy, a SSc patient without PAH and a healthy donor are shown.

Serum endothelial markers. Serum levels of soluble vWF (Figure 3a) were increased in both SSc-PAH patients (1043 mU/ml ±97) and SSc patients without PAH (1061 mU/ml ±86) in comparison with the HD group (770 mU/ml ±54, p<0.05) and did not change after 12 months of bosentan therapy (980 mU/ml ±89, not significant vs baseline). Serum levels of sP-selectin (Figure 3b) were significantly higher in the SSc-PAH group (367 ng/ml ±42) and in SSc patients without PAH (362 ng/ml ±61) than in HD (312 ng/ml ±12, p<0.01), and significantly decreased after 12 months of bosentan therapy (211 ng/ml ±31, p<0.05, vs baseline). Soluble PECAM serum levels were similar in SSc-PAH patients (48.7 ng/ml ±3), in SSc patients without PAH (46.7 ng/ml ±4) and in HD (41.1 ng/ml ±2) and were not modulated by bosentan treatment (37.7 ng/ml ±4). Serum levels of sVCAM-1 (Figure 3c) were significantly higher in SSc patients without PAH (300 ng/ml ±16) than in the HD group (261 ng/ml ±9, p<0.05), and were also increased in SSc-PAH patients (303 ng/ml ±3) but without reaching statistical significance. However, sVCAM-1 levels significantly dropped after 12 weeks of bosentan therapy (229 ng/ml ±15, p<0.01 vs baseline). Furthermore, sICAM-1 serum levels (Figure 3d) were significantly higher in SSc-PAH patients (2536 pg/ml ±647) and SSc patients without PAH (3293 pg/ml ±1006) than in HD (588 pg/ml ±48, p<0.01), and declined to normal levels following bosentan treatment (696 pg/ml ±98, p<0.05 vs baseline). Finally, soluble PECAM levels (Figure 3e) were increased in SSc-PAH patients (46.7 ng/ml ±4) in comparison with HD (41 ng/ml ±2), but the difference was not statistically significant, and significantly reduced after bosentan treatment (37 ng/ml ±3, p<0.05).

No significant correlations were found between adhesion molecules, either soluble or expressed on T cells, and clinical parameters such as modified Rodnan skin score, 6-MWD, NYHA classes, new digital ulcers, FVC, DLCO. Furthermore, no correlations between adhesion molecule changes over time and treatment response were detected.
DISCUSSION

The pathophysiology of SSc is multifaceted and different types of cells, such as fibroblasts, immune cells, inflammatory cells, endothelial cells, smooth muscle cells, pericytes, myointimal cells, are involved. Each disease picture is unique because the pathological process may lead to different tissue damage depending on the organ involved. In recent years, the study of pulmonary vasculature involvement has posed a strong challenge since pulmonary arterial hypertension (PAH) dramatically affects the clinical outcome and survival of SSc patients, especially those classified within the limited cutaneous subset.

A widely accepted hypothesis is that, maybe on a genetic basis and following unknown external stimuli, endothelial cells and lymphocytes become activated and stimulate fibroblasts and smooth muscle cells to proliferate and produce collagen and other ECM components. Endothelial damage would let leukocytes migrate through the vessels into the extra-vascular tissue and promote the activation of the innate immunity. This local inflammatory response would be self-limiting without the transition to the adoptive immunity with activation of T cells that sustain a chronic inflammatory state and stimulate fibrosis by both directly interacting with fibroblasts and producing fibrogenic mediators, such as IL-4, IL13, TGF-β1. Focusing on the interplays between endothelial cells and lymphocytes may be crucial to unravel the early events occurring in SSc pathogenesis. Circulating T cells interact with endothelial layer through an intricate system of receptors and their ligands, generally known as “adhesion molecules”. L-selectin and P-selectin, oligosaccharide proteins belonging to the family of selectins, are expressed on T lymphocytes and endothelial cells, respectively, and reciprocally interact to allow loose adhesion of circulating lymphocytes. If a firm adhesion, which preludes migration through the vessels into the extra-vascular space, occurs, endothelial cells and T cells undergo activation and modulate the expression of their surface receptors. ICAM-1 (intercellular adhesion molecules-1) and VCAM-1 (vascular adhesion molecules-1) are up-regulated on activated endothelial cells and interact with LFA-1 (lymphocyte function antigen-1) and VLA-4 (very late antigen 4), respectively, expressed on activated T cells. When over-expressed on the cell surface, some of these proteins are shed in biological fluids, as a regulatory mechanism, and the soluble forms, sVCAM-1, sICAM-1, sP-selectin, can be dosed and used as markers of endothelial cell activation. Many authors have shown that serum activation markers are increased in SSc patients, and have attempted to correlate them to disease activity.

Among the array of cytokines involved, ET-1, mainly released by endothelial cells, has been shown to play a physiological role in controlling vascular tone by regulating both vasodilatation and vasoconstriction. In SSc, changes of crosstalk between ET-1 and its receptors due to a different distribution of ET_A and ET_B receptors break down this balance, causing vasoconstriction to predominate. Besides vascular tone alterations, ET-1 stimulates smooth muscle cells and myofibroblasts and promote vasoproliferative changes leading to increased pulmonary vasculature resistances and subsequent PAH. The importance of ET-1 in the pathogenesis of PAH is further corroborated by the beneficial effects of the dual endothelin receptors inhibitor, bosentan, in the treatment of PAH.

In this study we evaluate the expression of LFA-1, VLA-4, and L-selectin antigens on circulating T cells and serum levels of soluble vWF, ICAM-1, VCAM-1, and P-selectin in SSc patients with associated PAH, at baseline and after 12 months of bosentan therapy, as compared with SSc patients without PAH and healthy subjects. Our results confirm that bosentan treatment improves exercise ability and dyspnea in SSc-PAH patients and provide further evidence that endothelial activation occurs in SSc, since soluble endothelial activation markers were significantly higher in the whole SSc group than in controls. Interestingly, whereas vWF antigen levels did not change after bosentan therapy, serum levels of sICAM-1, sVCAM-1, sPECAM and sP-selectin fell to normal values following blocking of the ET_A and ET_B receptors. These findings are apparently in contrast with those recently reported by Sfikakis et al who did not detect changes in sICAM-1.
levels in SSc patients following bosentan treatment. However, the latter was a short term study in which sICAM-1 was assessed in 10 SSc patients treated with bosentan at lower dosage for 4 months.

The behavior of T cell subsets bearing adhesion molecules was noteworthy. T cells expressing LFA-1, the counter-receptor of ICAM-1, were significantly and selectively enhanced in the SSc-PAH group at baseline, and LFA-1 expression was down-regulated by bosentan therapy. The reduction of VLA-4 on T cells from SSc-PAH patients was unforeseen as its expression is expected to increase on activated T cells and the meaning of these findings is unknown. VLA-4 has pleiotropic functions and it is involved in T cells/endothelial cells interactions as well as in T cells/stromal cells interplay. The finding that VLA-4 was selectively reduced on peripheral T cells from patients with SSc-PAH but not in SSc patients without PAH and HD, suggests that this molecule may be directly involved in SSc-PAH pathogenesis. However, since bosentan did not modify VLA-4 expression on T cells, it is conceivable that ET-1 does not regulate VLA-4. The pattern of L-selectin was remarkable in that its expression on T cells was lower in SSc-PAH patients than in healthy donors or SSc patients without PAH, and strikingly increased to normal values after bosentan treatment. These data suggest that bosentan, a dual endothelin receptor antagonist approved for the therapy of PAH, is able to restore T cell functions in SSc patients with PAH “in vivo”. We can speculate that blocking both ET-1 receptors can antagonize the detrimental effects of the imbalance of the ET-1 system in SSc. How inhibition of ET-1 receptors may regulate LFA-1 and L-selectin expression on circulating T cells can only be a matter of speculation at the moment. This may be indirectly mediated by the microvasculature, which expresses both ET\textsubscript{A} and ET\textsubscript{B} receptors, and changes of adhesion molecules on endothelial cells might modify the expression of their respective ligands on circulating T cells. Additionally, it may be assumed that T-cells may bear ET-1 receptors on their membrane and that the changes of LFA-1 and L-selectin expression we detected in our SSc-PAH patients are due to down-stream signals following direct stimulation by ET-1, subsequently inhibited by bosentan. This hypothesis needs to be verified by further studies “in vitro” addressed to show the expression of ET-1 receptors by T cells and the role of ET-1 in modulating T cell phenotype. However, an analogous mechanism has already been shown on human neutrophils. ET-1 down-regulated the expression of L-selectin and up-regulated the expression of LFA-2 on neutrophils and these effects were selectively prevented by a specific ET\textsubscript{A} receptor antagonist. These data seem to be consistent with our results and coherent with the sequential two-step action of these molecules. Resting leucocytes highly express L-selectin and loosely adhere to endothelial cells, the so called “tethering”, and roll on the endothelial layer. After activation, endothelial cells up-regulate ICAM-1 and leucocytes down-regulate L-selectin and simultaneously increase LFA-1 expression. LFA-1/ICAM-1 interactions would lead leucocytes to firmly adhere and migrate through the vessels. Furthermore, LFA-1/ICAM-1 ligation may be involved in T cells/fibroblasts interactions. In SSc-PAH, ET-1 could stimulate these events on T cells, which become activated, with high LFA-1 and low L-selectin expression, and migrate in the extra-vascular space, while bosentan down-modulates these processes and prevents further priming of circulating naive T cells. However, it should be taken into account that dermal fibroblasts may be a further source of increased serum levels of adhesion molecules. ICAM-1 expression on surface fibroblasts is modulated by ET-1 and inflammatory cytokines, is involved in cell-cell and cell-matrix interactions and plays a key role in regulating inflammatory cells binding to dermal tissue.

In conclusion, this study provides evidence that ET-1 can induce changes in the T-cell/endothelium interplay in SSc-associated PHA and that blocking ET-1 by the administration of bosentan can restore these interactions.
Table 1. Patients characteristics. Mean (range).

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<td>Age, yrs</td>
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<td>FVC/DLCO ratio</td>
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Legends to figures.

**Figure 1.** Percentage of peripheral blood T cells expressing LFA-1 (lymphocyte function antigen-1) (a), VLA-4 (very late antigen-4) (b), and L-selectin (c) in healthy donors (HD), systemic sclerosis patients without pulmonary arterial hypertension (SSc) and systemic sclerosis patients with pulmonary arterial hypertension (SSc-PAH) at baseline (t 0), after 6 months (t 6), and 12 months (t 12) of bosentan therapy. Mean ± 1 SD.

**Figure 2.** Fluorescence intensity for CD3-L-selectin cell subset from a SSc-PAH patient at baseline and after 10 months of bosentan therapy (upper panels), from a SSc patient without PAH (middle panels), and from a healthy donor (lower panels). Negative control histograms (a fluorescent non-binding mAb) are shown on the left. The longitudinal axis shows the percentage of lymphocytes positive for CD3 and L-selectin and the horizontal axis the mean channel fluorescence.

**Figure 3.** Serum levels of soluble von Willebrand Factor (vWF) (a), P-selectin (b), VCAM-1 (vascular cell adhesion molecule 1) (c), ICAM-1 (intercellular adhesion molecule-1) (d), and PECAM (platelet endothelial cell adhesion molecule) (e) in healthy donors (HD), systemic sclerosis patients without pulmonary arterial hypertension (SSc) and systemic sclerosis patients with pulmonary arterial hypertension (SSc-PAH) at baseline (t 0), and after 12 months (t 12) of bosentan therapy. Mean ± 1 SD.
Acknowledgements
This work was supported by grants from the University of Bari, Italy

Reference List


Fig. 1a

Percentage of CD3-LFA1 T cells

- HD
- SSc
- SSc-PAH t 0
- SSc-PAH t 6
- SSc-PAH t 12

- p < 0.05
- p < 0.05
- p < 0.05
Percentage of CD3-L-selectin T cells

HD SSc SSc-PAH t 0 SSc-PAH t 12

p<0.01

p<0.01

p<0.01

p<0.05

p<0.05

Fig. 1c
Fig. 3a
Fig. 3b
Soluble VCAM-1 ng/ml

HD  SSc  SSc-PAH t 0  SSc-PAH t 12

Fig.3c
Fig. 3d
Fig. 3e

Soluble PECAM ng/ml

HD  |  SSc  |  SSc-PAH t 0  |  SSc-PAH t 12

p<0.05  p<0.05
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